REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
May 23, 1995

3. REPORT TYPE AND DATES COVERED

4. TITLE AND SUBTITLE
Differential Gene Expression in Mammalian Liver During Hibernation

5. FUNDING NUMBERS
DAAL03-92-G-0019

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
ARO 29345.3-LS

11. SUPPLEMENTARY NOTES
The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words) Hibernation in mammals leads to profound reductions in metabolic, heart, and respiratory rates, as well as body temperature. The physiological events that lead to hibernation are precisely controlled, yet little is known about how these events are orchestrated at the molecular level. The first step toward a detailed molecular understanding of hibernation is to isolate and identify gene products that are uniquely expressed during hibernation. Liver was chosen to begin such a study because of its primary role in homeostasis; this role is likely to be critical during the physiological extremes experienced during hibernation. We used both a specific and a general approach to examine liver gene expression during hibernation. Alpha-2-macroglobulin, a broad-spectrum protease inhibitor, is expressed at elevated levels during hibernation. Because alpha-2-macroglobulin is an acute phase reactant in some species, we asked whether the acute phase response of the liver is a normal part of the biochemical events necessary for hibernation. In order to examine the general features of differential gene expression in the liver during hibernation, we used a subtractive hybridization approach to isolate and characterize cDNAs corresponding to genes that are induced in the hibernating state.

14. SUBJECT TERMS
Liver, Gene Expression, Hibernation, Ground Squirrels, Acute Phase Response, Mitochondria, Oxidative Phosphorylation alpha-2-macroglobulin

15. NUMBER OF PAGES
5

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Title Page

a) Proposal Number: 29345-LS.

b) Period Covered by Report: February 1, 1992- March 31, 1995.

c) Title of Proposal: **Differential Gene Expression in Mammalian Liver During Hibernation.**

d) Contract/Grant Number: DAAL03-92-G-0019.

e) Name of Institution: University of Colorado Health Sciences Center

f) Author of Report: Sandra L. Martin, PhD

g) List of Manuscripts Submitted or Published Under ARO Sponsorship During this Reporting Period and Invited Presentations:

Srere, H.K., Wang, W.H.C., and Martin, S.L. (1992) A Central Role for Differential Expression During Mammalian Hibernation. **Proc. Natl. Acad. Sci., USA** 89:7119-7123.

Martin, S.L., Srere, H.K., Belke, D., Wang, L.C.H., and Carey, H.V. (1993) Differential Gene Expression in the Liver during Hibernation in Ground Squirrels. *in Life in the Cold III: Ecological, Physiological and Molecular Mechanisms*. Carey, C., Florant, G.L., Wunder, B.A. and Horwitz, B., eds. Westview Press, Boulder, Colorado, 443-453.

Thomas, W.K. and Martin, S.L. (1993) A Recent Origin of the Marmot, **Mol. Phylogen. Evol.** 2, 330-336.

Srere, H.K., Belke, D., Wang, L.C.H., and Martin, S.L. (1995) a_2-Macroglobulin Gene Expression is Independent of the Acute Phase Response during Hibernation in Ground Squirrels. **Amer. J. Physiol.**, in press.

Invited Speaker, Plenary Lecture, Life in the Cold III (International Symposium on Adjustments of Vertebrates to Cold). Crested Butte, CO, fall 1993.

Invited Speaker, Plenary Lecture, Experimental Biology, '94. Anaheim, CA, spring 1994.

Invited Discussant, APS Intersociety Meeting: Regulation, Integration and Adapatation: A Species Approach. San Diego, CA, fall 1994.

h) Scientific Personnel Supported: Sandra L. Martin, PhD, PI; Hilary K. Srere, PhD, Doctoral Student; Stephanie A. Trelogan, Doctoral Student.

I) Report of Inventions by Title: None.

Differential Gene Expression in Mammalian Liver During Hibernation: Final Progress Report.

The long-range goal of the supported work was to understand what molecular changes, especially at the level of differential gene expression, have occurred during evolution that allow some mammals to hibernate. The first step towards this goal is to understand the molecular mechanisms that underlie hibernation in at least one species. Mammals experiencing the dramatic physiological extremes in body temperature and metabolic rate that accompany the adaptation of hibernation face a severe challenge to maintain internal homeostasis. Since the liver plays a key role in the maintenance of homeostasis during normothermia, liver function is also likely to be an important component of survival during hibernation. In order to identify liver genes whose products play a role during hibernation, we followed two courses: 1) examine the expression of a set of genes known as the acute phase reactants that our preliminary studies had suggested may increase in concentration during hibernation in ground squirrels, and 2) isolate and characterize liver genes whose expression changes as a function of hibernation using a method that makes no assumptions regarding their identity at the outset.

1) Role of the Acute Phase Response During Hibernation. Our previous work identified a plasma protein whose levels are elevated during hibernation as α_2-macroglobulin, a broad-spectrum protease inhibitor that is also elevated as part of the acute phase response in some species (Gehring *et al.*, 1987; Sehgal *et al.*, 1989). Its induction during hibernation raised the testable hypothesis that elicitation of the acute phase response is a standard component of the molecular events involved in hibernation.

The acute phase response involves a complex repatterning of liver gene expression. While total protein synthesis remains quantitatively similar to that in normal liver, there are significant qualitative changes in the proteins produced. The expression of some genes is reduced, whereas the expression of other genes is increased. The types of proteins that are induced during the acute phase response include proteinase inhibitors, clotting factors and transport plasma proteins. In general, the acute phase response is thought to play an important defensive role in the ability of an organism to withstand tissue damage and infection (Sehgal *et al.*, 1989; Fey and Gauldie, 1990 and references therein). Thus, elicitation of the acute phase response might play a protective role for a mammal experiencing the physiological extremes of hibernation. Since there is variation from species to species in the specific proteins that are induced during the acute phase reaction (usually explained by the fact that many of the plasma proteins play multiple, sometimes redundant, roles), it was important not only to define the acute phase response of a ground squirrel during inflammation, but also to examine the expression of acute phase reactants other than α_2-macroglobulin during hibernation.

The acute phase response was induced in Richardson's ground squirrels by injection of Freund's adjuvant. Following this treatment, an increase in α_2-macroglobulin mRNA and its corresponding protease inhibitor activity was observed; thus, we concluded that α_2-macroglobulin is a minor acute phase reactant in this species. The mRNA levels of several other well-

characterized acute phase reactants were also examined following injection with Freund's adjuvant. α_1-Antitrypsin, C-reactive protein, ceruloplasmin, serum amyloid A and serum amyloid P all show significant elevation in our model acute phase induction, although none of them increased in amount during hibernation. Taken together, these results indicate that α_2-macroglobulin is independently regulated for hibernation in ground squirrels; i.e., the acute phase response is not induced as a normal component of hibernation (Srere *et al.*, 1995).

A second aspect of this work addressed the possible physiological role of elevated α_2-macroglobulin during hibernation. α_2-Macroglobulin appears to be the main physiological inhibitor of activated factor X in the mouse (Imber and Pizzo, 1981; Fuchs and Pizzo, 1983), and, therefore, is thought to play a key role in the control of blood clotting *in vivo*. For this reason, we assayed clotting time in plasma taken from active and hibernating Richardson's ground squirrels. When assayed at either 5°C or at 37°C, clotting time is significantly increased in the plasma isolated from hibernating animals (Srere *et al.*, 1995). This observation is consistent with the observed elevation in α_2-macroglobulin in the plasma of hibernating ground squirrels. The reduced clotting efficiency during hibernation makes physiological sense because reduced blood flow in the capillary beds of hibernating animals increases the likelihood of peripheral clotting.

2) Hibernation-Specific Genes: Changes in Liver Gene Expression during Hibernation. The goal of this objective was to isolate and characterize cDNA clones that correspond to genes whose expression is increased during hibernation.

A strategy was chosen that allowed enhancement of our hibernating liver cDNA library for a subset of cDNA clones corresponding to mRNAs that are either enriched or newly expressed during hibernation. The approach also simultaneously depletes the library of inserts whose mRNAs are expressed at elevated levels in the active state. The method employed was a subtractive hybridization strategy (Owens *et al.*, 1991) using cDNA libraries that we created from mRNA isolated from active and hibernating Richardson's ground squirrel liver. After three rounds of subtraction of the hibernating library (using driver RNA prepared from the active cDNA library), the remaining hibernation-specific sublibrary was 97.6% subtracted, based on removal of albumin clones. From within this sublibrary of about 10,000 clones, 201 of them became the foci for more detailed analysis. These were selected because of their ability to hybridize to a subtracted, hibernation-specific cDNA probe (Travis and Sutcliffe, 1988), but not to an active cDNA probe.

Inserts were examined for cross-hybridization to one another, which is indicative of independent cloning events from a single mRNA species; those that cross-hybridized were placed into groups. Representative inserts from these groups were used as probes on Northern blots to test whether the corresponding RNA was actually induced. A partial DNA sequence was also determined for several inserts. The largest group of inserts (49 of the cDNA isolates) appeared to correspond to subunit II of ground squirrel cytochrome *c* oxidase. Other, smaller groups, as well as several individual clones that came through the subtraction, also contained inserts related to mitochondrial function and were confirmed to be induced at the RNA level during hibernation.

Among these are ATP synthase, cytochrome *c* oxidase-subunit I, F1-ATPase, and NADH-ubiquinone oxireductase. Only one cDNA was isolated and characterized in this study that did not function in mitochondrial energetics--polyA polymerase (See Table 1).

Table 1. cDNA Clones Isolated by Subtractive Hybridization and Confirmed to be Induced during Hibernation

Clone	# Cross-hybridizing	Match in GenBank
1B6	none	F1-ATPase
1G7	none	polyA polymerase
2B8	none	cytochrome c oxidase, subunit I
1G3	five	ATP synthase
1B10	eight	NADH-ubiquinone oxireductase
2B11,4D8	forty-eight	cytochrome C oxidase, subunit II

In **SUMMARY**, our work during the past funding period led to the finding that α_2-macroglobulin, an acute phase reactant in some species, is elevated during hibernation in ground squirrels (Srere *et al.*, 1992; Martin *et al.*, 1993). A careful examination of the acute phase response in Richardson's ground squirrel, however, has led to rejection of the hypothesis that the acute phase response of the liver is part of the normal molecular strategy of hibernation. Instead, α_2-macroglobulin may be induced during hibernation for the purpose of slowing the rate of blood clotting. This would serve to reduce the probability of clotting in the capillary beds during hibernation, when heart rates are dramatically reduced (Srere *et al.*, 1995). In addition, we have provided evidence for induction of a number of gene products that function in mitochondria, suggesting a heretofore-unrecognized importance of liver mitochondrial function during hibernation (Srere and Martin, 1995). Finally, our work has led to recognition of the possibility that arousal, at least in part, may be necessary for the biosynthesis of gene products that are important for hibernation and its survival (Martin *et al.*, 1995).

Reference List: Please note that publications resulting from work supported by ARO during the previous funding period are marked with an asterisk.

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